JC08 Rec'd PCT/PTO 26 APR 2001

FORM PTO-1390 (Modified) (REV 5-93) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: 033236/0114

09/830343

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5): Unassigned

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/GB99/03553	October 27, 1999	October 27, 1998

TITLE OF INVENTION
LHRH ANALOGUES FOR THE TREATMENT OF OSTEOPOROSIS

APPLICANT(S) FOR DO/EO/US
Karen AKINSANYA, Amanda HAYWARD and Steve QI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.

3. ☐ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   - ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ has been transmitted by the International Bureau.
   - ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   - ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - ☐ have been transmitted by the International Bureau.
   - ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).

9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

11. ☐ Applicant claims small entity status under 37 CFR 1.27 .

Items 12. to 17. below concern other document(s) or information included:

12. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

13. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

14. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

15. ☐ A substitute specification.

16. ☐ A change of power of attorney and/or address letter.

17. ☐ Other items or information:

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U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Unassigned 09/830343	PCT/GB99/03553	033236/0114

18. ☒ The following fees are submitted:

	CALCULATIONS	PTO USE ONLY
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....$860.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2) $710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO $1,000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$860.00	
Surcharge of $130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))		

Claims	Number Filed		Included in Basic Fee		Extra Claims		Rate	
Total Claims	12	-	20	=	0	×	$18.00	$0.00
Independent Claims	5	-	3	=	2	×	$80.00	$160.00
Multiple dependent claim(s) (if applicable)							$270.00	
TOTAL OF ABOVE CALCULATIONS =								$1020.00
Reduction by ½ for filing by small entity, if applicable.								$0.00
SUBTOTAL =								$1020.00
Processing fee of $130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f). +								
TOTAL NATIONAL FEE =								$1020.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). $40.00 per property +								
TOTAL FEES ENCLOSED =								$1020.00
							Amount to be: refunded	$
							charged	$

a. ☒ A check in the amount of $1020.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 19-0741 in the amount of $1020.00 to the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0741. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE

NAME STEPHEN A. BENT

REGISTRATION NUMBER 29,768

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 033236/0114

In re patent application of

AKINSANYA, KAREN *et al.*

Serial No. 09/830,343

Filed: April 26, 2001

For: LHRH ANALOGUES FOR THE TREATMENT OF OSTEOPOROSIS

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents
Washington, D.C. 20231
Box SEQUENCE

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

9.2001
Date

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VERIFICATION SUMMARY DATE: 12/19/2001
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L:125 M:341 W: (46) "n" or "Xaa" used, for SEQ ID#:7

LHRH ANALOGUES FOR THE TREATMENT OF OSTEOPOROSIS

FIELD OF INVENTION

The present invention relates to pharmaceutical compositions for the treatment of osteoporosis and other disorders of bone metabolism.

BACKGROUND TO THE INVENTION

i) Osteoporosis

Osteoporosis is a disease that affects many elderly people. Post-menopausal osteoporosis associated with oestrogen deficiency is the most common bone disease in the western world (Melton, J. Bone Miner. Res. 10 174-177, 1995). The basic mechanism underlying osteoporosis is when bone resorption exceeds bone formation resulting in net bone loss and an increase in the incidence of fractures. The dynamic processes that control bone morphogenesis take place in bone and bone marrow. Bone is a hard calcified connective tissue consisting of osteoprogenitor cells, osteoblasts, osteocytes and osteoclasts. Bone marrow is composed of haematopoietic stem cells and mesenchymal cells. These cells include osteoprogenitors, chondrocytes, adipocytes, fibroblasts and reticular cells. Bone metabolism and remodelling is dependent on a balance between two processes; bone resorption is dependent on osteoclast activity and bone formation is dependent on osteoblast activity. Osteoblast precursors are fibroblast-like, proliferating progenitor cells, which differentiate into osteoblasts that synthesise and deposit matrix to become new bone. Osteoclasts are a tissue specific subtype of macrophages responsible for resorption of bone. Mononuclear phagocytes from bone marrow fuse to form the large multinucleated osteoclasts. Osteoclastogenesis is a complex phenomenon that is facilitated by the interaction of bone marrow stromal cells with haematopoietic osteoclast precursors (Suda *et al.*, Endocr. Rev. 13 66-80, 1992).

A number of factors regulate the proliferation and differentiation of osteoblasts and osteoclasts including cell-cell interactions, cytokines, growth factors, steroid hormones and neurohormones. These factors act via endocrine, autocrine and paracine mechanisms. Parathyroid hormone (PTH) is a key systemic regulator of calcium and bone metabolism acting principally by regulating bone resorption. There is evidence to suggest that PTH, vitamin D and calcitonin actions may be mediated by local factors found in bone (Oursler *et al.*,

Endocrinol. 129 3313-3320, 1990). A number of growth factors have been implicated such as insulin-like growth factors (IGF-I and IGF-II), fibroblast growth factors (bFGF and aFGF) (Baylink *et al.*, J. Bone Mineral Res. 8 (Supp. 2) S565-S572, 1993). Identification of locally produced agents that influence the process of bone formation and bone resorption may provide alternatives to current treatments for osteoporosis which include salmon calcitonin and hormone (oestrogen and progesterone) replacement therapy (Lindsay *et al.*, Lancet 341 801-805, 1993).

ii) GnRH-II

Studies on the physiology of the hypothalamic-pituitary-gonadal axis have resulted in the recognition of gonadotropin releasing hormone (GnRH, otherwise known as luteinizing hormone releasing hormone, LHRH) as a key regulatory hormone. GnRH is released by the hypothalamus and acts on the pituitary to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). More recently, a peptide related to GnRH has been identified, first in chickens (Miyamato, US Patent No. 4,540,513) and subsequently in humans (White *et al.*, Proc. Natl. Acad. Sci. USA 95 305-309, 1998). This peptide has been called GnRH-II. The sequences of the two peptides are compared below.

GnRH pyroGlu-His-Trp-Ser-**Tyr**-Gly-**Leu-Arg**-Pro-Gly-NH_2 (5)
GnRH-II pyroGlu-His-Trp-Ser-**His**-Gly-**Trp-Tyr**-Pro-Gly-NH_2 (6)

Analogues have also been reported (Volkers *et al.*, US Patent No. 4,721,775). No clear evidence for a physiological role for GnRH-II has so far been presented.

SUMMARY OF THE INVENTION

We have now found that GnRH-II is capable of modulating the differentiation of bone precursor cells and inducing the expansion of osteoblast populations. Accordingly, it is an object of the present invention to provide a pharmaceutical composition for the treatment of osteoporosis, which composition is characterised by the inclusion of GnRH-II or an analogue thereof. More specifically, the composition includes a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

wherein **Xaa**[1] is His or Tyr,
Xaa[2] is Trp or Leu, and
Xaa[3] is Tyr or Arg,

provided that when **Xaa**[1] is Tyr and **Xaa**[2] is Leu, then **Xaa**[3] is not Arg. The invention further comprises a method for the preparation of such a composition, wherein the active peptide is combined with pharmaceutically acceptable excipients, and a method of treatment of osteoporosis, which is the administration to an individual in need of such treatment a therapeutically effective amount of the composition.

DESCRIPTION OF THE FIGURE

Figure 1 shows the effect of increasing doses of GnRH-II on serum calcium concentrations in ovariectomised rats.

DESCRIPTION OF THE INVENTION

As used herein, abbreviations referring to amino acids have their conventional meanings and indicate the natural L-isomer (except for the achiral amino acid glycine).

In a first embodiment, the invention as disclosed herein comprises a pharmaceutical composition for increasing bone mass or bone density, or for accelerating bone growth or repair. Preferably, the invention as disclosed herein comprises a pharmaceutical composition for the treatment of osteoporosis (including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis). The composition is characterised in that it includes as an active principal a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**[1]-Gly-**Xaa**[2]-**Xaa**[3]-Pro-Gly-NH_2 (7)

wherein **Xaa**[1] is either His or Tyr, **Xaa**[2] is either Trp or Leu, and **Xaa**[3] is either Tyr or Arg, provided that when **Xaa**[1] is Tyr and **Xaa**[2] is Leu, then **Xaa**[3] is not Arg. Preferably, **Xaa**[1] is His, **Xaa**[2] is Trp, and **Xaa**[3] is Tyr. It will be recognised that such a peptide can form salts

with acids (for example, acetic acid, trifluoroacetic acid, benzoic acid, hydrochloric acid, phosphoric acid and the like). To the extent that such salts are formed with pharmaceutically acceptable acids, they are included within the scope of the invention.

It will be understood by those familiar with the art that the composition will also comprise one or more excipients, such as diluents, carriers, preservatives, and the like. The composition may be a solid or liquid formulation, and will be appropriate for the intended route of administration. For example, the composition might be formulated as a tablet or capsule for oral administration, or as a suppository for rectal or vaginal use. Equally, the composition might be formulated as a solution, for example in isotonic saline, for intravenous administration. The composition may also be presented as a kit for formulation immediately prior to administration, for example as a freeze-dried powder in a sealed ampoule supplied with a second ampoule containing a solvent such as saline.

The composition may be used in clinical situations other than for the treatment of osteoporosis, where bone mass or bone density are reduced, or where bone growth or repair needs to be accelerated. Such situations include (but are not limited to) the treatment of osteogenesis imperfecta and osteomalacia, the prevention of bone loss when an individual is immobilised for an extended period, bone segmental defects, periodontal disease, metastatic bone disease, osteolytic bone disease, other conditions requiring healing or regeneration of cartilage defects or injury, facial reconstruction procedures, and the facilitation of healing following a fracture. The compounds of the present invention may also be used in conjunction with agents that affect bone resorption, for example antiresorptive agents, such as estrogen, bisphosphonates and calcitonin. In addition, or alternatively, the compounds of the present invention may modulate calcium metabolism, cell proliferation and/or differentiation of normal or aberrant cells or tissues involved in bone physiology.

In a second embodiment, the invention disclosed herein comprises a method for the preparation of a pharmaceutical composition for the treatment of osteoporosis (including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis) or another disorder, which method comprises the mixing of a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

wherein **Xaa**^{1}, **Xaa**^{2} and **Xaa**^{3} are as defined above, with one or more pharmaceutically acceptable excipients. In the context of this invention, mixing is taken to include the blending together of solids, the dissolution of one or more solids in a liquid, and the dispersion of a solid in a liquid. The method may also include such processes as freeze-drying and microencapsulation necessary to obtain the formulation to be presented. Such processes as are known in the art are included within the scope of the present invention.

In a third embodiment, the invention as disclosed herein comprises a method for the treatment of an individual suffering from osteoporosis (including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis) or another bone disorder, or considered to be at risk of so suffering. This method of treatment comprises the administration to said individual of a therapeutically effective amount of a composition containing, as an active principal, a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

wherein **Xaa**^{1}, **Xaa**^{2} and **Xaa**^{3} are as defined above. The method of treatment may comprise a single administration of the composition, but is more likely to comprise a course of repeated administrations. The frequency of the administrations may be from once per month to four times per day. The amount of active peptide in each dose will depend on the dosing schedule and the route of administration. Generally, it will be between one microgram (1μg) and one hundred milligrams (100mg). Doses in the range 1-100μg are preferred when the composition is administered by intravenous injection. Doses in the range 10μg - 10mg are preferred when the composition is administered as slow-release

depots. Doses in the range 1-100mg are preferred when the composition is administered orally. Furthermore, high doses and frequent administration will be preferred when the desired outcome is to increase bone mass while lower doses and less frequent administration are more suited to the maintenance of bone mass.

The peptides that comprise the active agents of the compositions of the present invention can be prepared by the methods generally known in the art. For example, the peptides may be prepared by solid-phase synthesis. This involves the sequential addition of amino acid residues to a resin-bound intermediate according to the following strategy.

1. Formation of resin-bound first intermediate

   PG-Aaa-OH + FG-Res → PG-Aaa-L-Res

   Aaa = amino acid

   PG = protecting group

   FG = functional group

   Res = polymeric resin

   L = linker group (-O- or -NH-)

2. Deprotection

   PG-Aaa-L-Res → H-Aaa-L-Res

3. Chain extension

   PG-Bbb-OH + H-Aaa-L-Res → PG-Bbb-Aaa-L-Res

4. Repeat steps 2 and 3 as necessary

   PG-Bbb-Aaa-L-Res → → → PG-Nnn-...-Bbb-Aaa-L-Res

5. Cleave/deprotect

   PG-Nnn-...-Bbb-Aaa-L-Res → H-Nnn-...-Bbb-Aaa-OH (or $-NH_2$)

In step one, a protected amino acid is reacted with a functionalised resin. The protecting group (PG) is most commonly *tert*-butyloxycarbonyl (Boc) or 9-fluorenylmethyloxycarbonyl (Fmoc). The functional group on the resin (FG) is commonly a chloroalkyl group, a hydroxyl group or an amine group. When FG is a chloroalkyl or hydroxyl group, the linker group (L) is an oxygen atom (-O-). When FG is an amine group, L is -NH-.

In step two, the protecting group (PG) is removed from the α-amino group. When PG is Boc, this can be accomplished by treating the resin with acids such as trifluoroacetic acid or hydrogen chloride in dichloromethane. When PG is Fmoc, the deprotection can be accomplished by treating the resin with bases such as piperidine.

In step three, the peptide chain is extended by one amino acid residue. A protected amino acid is coupled to the amine group liberated in step two. Many reagents are known in the art for achieving this conversion. One combination is dicyclohexylcarbodiimide (DCC) and hydroxybenzotriazole (HOBt). Generally, a base will also be necessary. Suitable bases include triethylamine and N,N-diisopropylethylamine. The solvent will generally be dichloromethane, dimethylformamide, or a mixture of these.

If the side chains of the amino acids (Aaa - Nnn) contain reactive groups (for example amino groups, carboxylic acid groups, hydroxyl groups) then these will need protecting. The protecting groups chosen for the side chains are generally those that are stable under the conditions required to remove the protecting group (PG) from the α-amino group. If PG is Fmoc, then the side chain protecting groups can conveniently be based on tert-butyl chemistry. On the other hand, if PG is Boc, then the side chain protecting groups can be based on fluorenylmethyl chemistry. Other protecting groups known in the art can also be used.

In step four, the deprotection/chain extension cycle is repeated until the desired peptide sequence has been constructed.

In step five, the completed peptide is liberated from the resin. Protecting groups are removed from the side chains either before or after the cleavage. When L is -NH-, the peptide liberated is in the form of the C-terminal amide. When L is -O-, the peptide liberated is often the C-terminal free acid and a second step is required to form the C-terminal amide.

The peptides may also be prepared by solution-phase synthesis, and this may be more convenient when large quantities of material are needed.

The above general description is further elaborated below in a number of examples. These are intended to illustrate certain aspects of the invention. They are not intended to be limiting in any way.

EXAMPLES

Example 1 - Synthesis of GnRH-II

1A. Preparation of resin-bound protected peptide.

pyroGlu-His(Bom)-Trp(CHO)-Ser(Bzl)-His(Bom)-Gly-Trp(CHO)-Tyr(Bzl)-Pro-Gly- ORes

This peptide was prepared using standard solid-phase methods starting from Boc-Gly-esterified Merrifield resin (60 g, 1 mmol/g). The synthesis was performed in a manual synthesizer, with a total solvent and reagent volume of 300 mL for each operation. The standard deprotection/wash/coupling protocol is summarised in Table 1.

Table 1

Step	Reagent	Time (min)	Number of operations
Deprotection of Boc	HCl / DCM*	60	1
Washing	DCM	2 - 4	3
Neutralisation	10% DIPEA / DCM	4	2
Washing	DCM	2 - 4	1
Coupling	Activated ester	60 - 120**	1 - 2
Washing	DCM	2 - 4	3

* Gaseous hydrogen chloride was bubbled through a suspension of the resin in DCM

** Completeness of reaction was determined by a negative ninhydrin test

Benzotriazolyl esters were used as the activated esters throughout the synthesis. These were prepared from the corresponding protected amino acids by reaction with 1-

hydroxybenzotriazole (1eq.) and dicyclohexylcarbodiimide (1eq.). The quantities used (in relation to the resin substitution capacity) are listed in Table 2.

Table 2

Cycle no.	Amino acid derivative	Molar excess
1	Boc-Pro-OH	1.8
2	Boc-Tyr(Bzl)-OH	1.8
3	Boc-Trp(CHO)-OH	1.8
4	Boc-Gly-OH	1.8
5	Boc-His(Bom)-OH	1.8
6	Boc-Ser(Bzl)-OH	2.0
7	Boc-Trp(CHO)-OH	2.0
8	Boc- His(Bom)-OH	2.0
9	pyroGlu-OH	2.0

Following the final coupling, the resin was washed with dichloromethane (3 × 3L) and dried under reduced pressure at +40°C to constant weight.

Amino acid analysis: Consistent with proposed sequence

1B. Cleavage and deprotection

pyroGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (6)

The peptidoresin prepared in Example 1A was placed in a linen bag in a pressure vessel. The vessel was then charged with gaseous ammonia to a final pressure of 4atm. After 72h the excess ammonia was vented and the resin was extracted with acetic acid (3×100mL) and ethanol (3×100mL). The combined extracts were degassed with nitrogen, 10% palladium-on-carbon was added, and the mixture was stirred under an atmosphere of hydrogen. When the reaction was complete (as judged by HPLC), the mixture was filtered and the filtrate was evaporated. The residue was purified by reverse-phase HPLC to give the title compound.

Example 2 - Analysis of the effects of GnRH-II and analogues on Osteogenic cell populations *in vitro*.

(a) Human osteoblasts were isolated from cancerous bone from orthopaedic surgery (Nilsson *et al.*, 1995) according to standard procedures known in the art. The bone explants were minced into small bone chips and then washed extensively in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1 Gibco, Paisley, U.K). These osteoblast like cells, Murine osteoblastic MC3T3-E1 cells and human clonal osteosarcoma cell lines MG-63 (non-mineralising) and SaOS-2 (mineralising osteosarcoma) were cultured in DMEM:F12, 1:1 with the addition of 10% fetal calf serum (FCS, Gibco), fungizone (500mg/l), gentamycin sulphate (50mg/l), L-glutamine (2mM) and l-ascorbic acid (100mg/l) in a humidified CO_2 chamber at 37°C.

(b) Human bone marrow stromal cells were isolated from bone fragments rinsed in phosphate-buffered saline. Bone marrow cells were collected and spun through a column of Ficoll Hypaque (Kimble *et al* J. Clin. Invest. 93 1959-1967, 1994). Cells at the interface were pelleted, counted and seeded into 75cm^2 flasks. The cells were incubated in a humidified CO_2 chamber at 37°C and the medium changed weekly. At confluence, the cells were harvested using trypsin EDTA and re-seeded in α-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FCS, Gibco), penicillin (100U/ml), streptomycin (100mg/ml), fungizone and L-glutamine (2mM).

(c) All cells were serum-starved for 48h before addition of GnRH-I and GnRH-II. Cells were placed in DMEM without phenol red (in order to avoid oestrogen-like effects of phenol red) containing 10% charcoal-stripped serum for 48h in 12 well plates. Dose dependent effects of GnRH-I and GnRH-II and analogues of the peptides were studied following the addition of peptides at final concentrations ranging from 10^{-9} to 10^{-6}M. 1mM dibutyryl cAMP was used as a control. The cells were incubated for 24, 48 and 96h with the peptide being replaced every 24h.

(d) To assess the effects of the peptides on cell proliferation, [^{3}H]thymidine was added at 1mCi/ml for an additional 24h and [^{3}H]thymidine incorporation was determined. Radioisotope incorporation was determined using a scintillation counter and the results were calculated as cpm/mg of total protein.

(e) Expression of osteoblastic differentiation markers was also determined (Tintut Y *et al,*. J Biol Chem 273 7547-53, 1998). Total RNA was isolated at several stages before treatment, at 24, 48, 72 and 96h after addition of peptides. Type I procollagen, osteopontin and 28S RNA (used as an internal control) expression was determined by Northern blot analyses. Alkaline phosphatase, matrix GLA protein, osteoclastin and GAPDH (as an internal control) were determined by RT-PCR with specific primers designed for each gene.

The peptides of the invention caused significant effects at concentrations below 100μM.

Example 3 - Analysis of the effects of GnRH-II and analogues on Osteoclast populations *in vitro*.

(a) Human clonal cell lines of osteoclast precursors (FLG 29.1) were used as an *in vitro* model of osteoclast differentiation (Gattei V *et al.*, Cell Growth Differ 7 753-63, 1996). In addition, co-cultures of FLG 29.1 and osteoblastic cells (Saos-2) were evaluated for migratory, adhesive, cytochemical, morphological, and biochemical changes. Dose dependent effects of GnRH-I and GnRH-II and analogues of the peptides were studied following addition at final concentrations ranging from 10^{9} to 10^{-6}M to FLG 29.1 cultures and to co-cultures. Parathyroid hormone was added as a control. Potentiation (or inhibition) of the differentiation of the preosteoclasts (fusion into large multinucleated elements) and a number of other factors were measured (Orlandini *et al,*. Cell Tissue Res. 281 33-42, 1995). These included:

1. Positive staining for tartrate-resistant acid phosphatase in FLG 29.1 cells
2. A decrease of the alkaline phosphatase activity expressed by Saos-2 cells
3. The appearance of typical ultrastructural features of mature osteoclasts in FLG 29.1 cells
4. 4.The release into the culture medium of granulocyte-macrophage colony stimulating factor.
5. To assess the effects the peptides on cell proliferation, [^{3}H]thymidine was added at 1mCi/ml for an additional 24h and [^{3}H]thymidine incorporation was determined as described above.

(b) Bone marrow cells removed from human bone fragments were cultured in the presence of 10nM 1,25-$(OH)_2$ vitamin D_3 for seven days to generate multinucleated osteoclasts using standard techniques known in the art (Takahashi *et al.*, Endocrinol 122 1473-1482, 1988).

The culture medium (α-MEM) was removed and replaced by a fresh phenol red free medium supplemented with antibiotics and 10% charcoal-stripped heat-inactivated FCS containing GnRH-I, GnRH-II or analogues, and the cultures were maintained for a further 24h. Floating cells were harvested and osteoclasts stained for tartrate-resistant acid phosphatase (TRAP) expression, a marker of osteoclast differentiation (Hughes *et al.*, Nat. Med. 2 1132-1135, 1996)

1. Cells were incubated in 0.2M acetate buffer, pH 4.7-5.0, containing tartaric acid and 2% naphthol AS-BI phosphate (dissolved at 20mg/ml in ethylene glycol monomethyl ether) for 15min at 37°C. The cells were then transferred to a second solution consisting of the same buffer and concentration of tartaric acid with 0.1% pararosanoiline chloride (hexazotised by mixing with an equal volume of 4% sodium nitrite for 5min at room temperature) for 10min at 37°C. This treatment causes a red cytoplasmic stain in cells expressing TRAP. Harris' hematoxylin was used as a nuclear counterstain.
2. Apoptotic multinulceated osteoclasts were identified by strong expression of TRAP, larger size than accompanying viable TRAP-positive cells. Confirmation of apoptosis was carried out using acridine orange stain. Viable osteoclasts were counted after fixation in 95% ethanol and TRAP hematoxylin staining, and apoptotic osteoclasts were expressed as a percentage of the total number of multinucleated osteoclasts (viable and apoptotic) in each culture well.

The peptides of the invention caused significant effects at concentrations below 100μM.

Example 4- Expression analysis of GnRH mRNA in osteogenic and osteoclast cell populations

Total RNA was extracted from cells cultured as described above:

1. osteoblast like cells, isolated from cancerous bone
2. murine osteoblastic MC3T3-E1 cells
3. MG-63 (non-mineralising)
4. SaOS-2 (mineralising osteosarcoma)
5. human bone marrow stromal cells
6. human FLG 29.1 osteoclast precursor cells
7. multinucleated osteoclasts generated from bone marrow

Expression of GnRH-I and GnRH-II was determined by RT-PCR using PCR primers outlined in SEQ I.D. No 1-4. The integrity of the cDNA generated was determined by assessing the relative level of actin amplification.

Example 5 - Effect of GnRH-II on bone mineral density in the ovariectomised rat

(a) Female adult (8 weeks old, 200-215g) Sprague Dawley rats were bilaterally ovariectomised (OVX). Animals were kept for 4 weeks post-delivery before commencing treatment. Purina rat chow (1.00% calcium, 0.61% phosphorous) and water were provided ad libitum. Each study consisted of 6 weight-matched groups (n = 8/group).

(b) Treatment started 4 weeks post-OVX. After 4 weeks, a baseline control OVX group was sacrificed (Group A). The remaining groups were injected once a day with vehicle (Group B), 1μg/kg body weight (Group C), 10μg/kg body weight (Group D), 100μg/kg body weight (Group E) of GnRH-II, and 80μg/kg body weight (Group F) of hPTH(1-34).

(c) All rats were weighed every fourth day and dosages adjusted for 50g increase in mean group weight. Rats were given alternate subcutaneous injections of calcein (30mg/kg) or tetracyclin (30mg/kg) in 2% sodium bicarbonate-saline, respectively to label mineralization surfaces on days 10, 19 and 26, following treatment with drug. Bone mineral density was assessed by dual energy x-ray absorptometry-DEXA). On day 28 serum calcium levels were determined by colorimetric assay using a commercial kit.

(d) Success of OVX was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of the uterine horns. Both legs were disarticulated at the hip. The left tibia and femur were cleaned of excess muscle and soft tissue and placed in 70% ethanol. The anterior eminence of the right tibia metaphysis was shaved with a razor blade, barely exposing bone marrow. Both right femur and tibia were then placed in 10% phosphate-buffered formalin for 24h and transferred to 70% ethanol.

Ovariectomised animals treated daily with 10 and 100μg/kg of GnRH-II and 80μg/kg PTH for 28days have pronounced hypercalcemia. Results are shown in Figure 1.

Example 6 - Cellular localisation of GnRH-II in paraffin sections of normal rat bone and human bone.

(a) Frozen and/or paraffin–embedded human and rat bone sections were fixed for 3-36h depending on size (3-5h at room temperature, then approx 24h at 4°C) and then soaked in 0.1M Tris + 5 % EDTA (12.11g + 50g EDTA) pH 7.3 until decalicified.

(b) Sections were then processed for antibody staining (rabbit polyclonal anti-GnRH-II antibody) using standard techniques.

Staining for GnRH-II was observed in platelets, megakaryocytes at the growth plate (especially proliferating chondrocytes). Some staining was also seen in the bone-forming cells particularly in active osetoblasts as well as new osteoid.

Overall, these results demonstrate that GnRH-II has a role in bone growth and calcium metabolism. Accordingly, it is useful as a therapeutic agent in diseases that are related to inadequate bone growth or loss of bone tissue.

SEQUENCE LISTING

INFORMATION FOR SEQ ID No 1.

(i) Sequence Characteristics

(a) Length: 19 bases

(b) Type: nucleic acid

(c) Strandedness: single

(d) Topology: linear

(ii) Molecule Type cDNA

(iii) Sequence description: SEQ ID No.1

CTG CAG CTG CCT GAA GGA G (1)

INFORMATION FOR SEQ ID No 2.

(i) Sequence Characteristics

(a) Length: 19 bases

(b) Type: nucleic acid

(c) Strandedness: single

(d) Topology: linear

(ii) Molecule Type cDNA

(iii) Sequence description: SEQ ID No.2

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INFORMATION FOR SEQ ID No 3.

(i) Sequence Characteristics

(a) Length: 21 bases

(b) Type: nucleic acid

(c) Strandedness: single

(d) Topology: linear

(ii) Molecule Type cDNA

(iii) Sequence description: SEQ ID No.3

ATT CTA CTG ACT TGG TGC GTG (3)

INFORMATION FOR SEQ ID No 4.

(i) Sequence Characteristics

(a) Length: 21 bases

(b) Type: nucleic acid

(c) Strandedness: single

(d) Topology: linear

(ii) Molecule Type cDNA

(iii) Sequence description: SEQ ID No.4

GGA ATA TGT GCA ACT TGG TGT (4)

CLAIMS

1. A pharmaceutical composition for the treatment of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis, and other disorders of bone metabolism, or for accelerating bone growth or repair, which composition is characterised by the inclusion of a peptide

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

or a salt thereof

wherein **Xaa**^{1} is His or Tyr,
Xaa^2 is Trp or Leu, and
Xaa^3 is Tyr or Arg,

provided that when **Xaa**^{1} is Tyr and **Xaa**^{2} is Leu, then **Xaa**^{3} is not Arg.

2. The composition according to Claim 1, wherein the composition is used for the treatment of osteoporosis.

3. The pharmaceutical composition according to Claim 1, wherein the peptide is

pyroGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (6)

4. The composition according to Claim 3, wherein the composition is used for the treatment of osteoporosis.

5. A method of preparing a pharmaceutical composition for the treatment of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis, or another disorder of bone growth, which method comprises the mixing of a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

or a salt thereof

wherein **Xaa**^{1} is His or Tyr,
Xaa^2 is Trp or Leu, and
Xaa^3 is Tyr or Arg,

provided that when **Xaa**^{1} is Tyr and **Xaa**^{2} is Leu, then **Xaa**^{3} is not Arg, with one or more pharmaceutically acceptable excipients.

6. The method of Claim 5, wherein the composition is for the treatment of osteoporosis.

7. The method of Claim 5, wherein the peptide is

pyroGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (6)

8. A method of treatment of an individual suffering from osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis, or another disorder of bone growth, or at risk of so suffering, which comprises the administration to said individual of a therapeutically effective amount of a pharmaceutical composition which includes a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

or a salt thereof

wherein **Xaa**^{1} is His or Tyr,
Xaa^2 is Trp or Leu, and
Xaa^3 is Tyr or Arg,

provided that when **Xaa**^{1} is Tyr and **Xaa**^{2} is Leu, then **Xaa**^{3} is not Arg, as an active principal.

9. The method of Claim 8, wherein the disorder of bone growth is osteoporosis.

10. The method of Claim 8, wherein the peptide is

pyroGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (6)

11. A new use for a peptide according to the sequence

pyroGlu-His-Trp-Ser-**Xaa**^{1}-Gly-**Xaa**^{2}-**Xaa**^{3}-Pro-Gly-NH_2 (7)

or a salt thereof

wherein **Xaa**^{1} is His or Tyr,
Xaa^2 is Trp or Leu, and
Xaa^3 is Tyr or Arg,

provided that when **Xaa**^{1} is Tyr and **Xaa**^{2} is Leu, then **Xaa**^{3} is not Arg,

which use is as a therapeutic agent for the treatment of osteoporosis, including age-related osteoporosis and osteoporosis associated with post-menopausal hormone status, primary and secondary hyperparathyroidism, disuse osteoporosis, diabetes-related osteoporosis, and glucocorticoid-related osteoporosis, and other disorders of bone growth.

12. A new use for a peptide according to the sequence

pyroGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 (6)

which use is as a therapeutic agent for the treatment of osteoporosis and other disorders of bone growth.

1/1

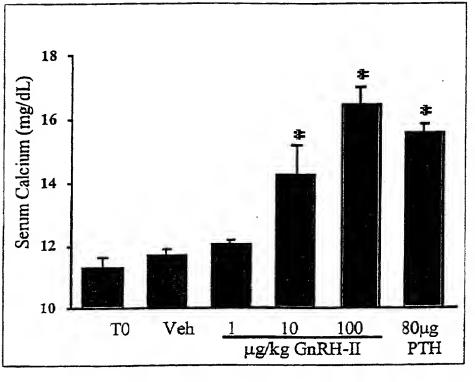
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Figure 1

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

LHRH ANALOGUES FOR THE TREATMENT OF OSTEOPOROSIS

(Attorney Docket No. 033236/0114)

the specification of which (check one)

___ is attached hereto.

__X__ was filed on PCT/GB99/03553 as United States Application Number or PCT International Application Number October 27, 1998 _ and was amended on _____ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code §119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?
9823515.3	Great Britain	October 27, 1998	Yes	

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date

I HEREBY CLAIM the benefit under Title 35, United States Code, §120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the following registered attorneys and agents of the law firm of FOLEY & LARDNER:

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to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.

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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Date	7/13/01

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Inventor's signature	
Date	07/13/01

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HAYWARD, AMANDA
QI, STEVE

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